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The Effects of Bisphenol F on Embryonic Cardiac Output in Zebrafish

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Introduction:

In the past two decades, there has been increasing awareness and concern about endocrine disrupting chemicals (EDCs) which pose a threat to development and reproduction in wildlife and human populations. Bisphenol A (BPA), a xenoestrogen that exhibits EDC activity, was a commonly used plasticizing agent used in epoxy-resins and in plastic food and beverage containers; however, widespread testing demonstrated detrimental effects of acute and chronic exposure to this additive (Eladak et al., 2015; Rezg et al., 2014; Duan et al,. 2008). Although the effect of BPA is systemic, specific changes have been observed to the cardiovascular system, including increased arrhythmia and improper calcium transport and storage mechanisms (Gao & Wang, 2014; Qiu et al., 2018). As a result of numerous studies, there has been a widespread push for the removal of BPA from commonly used household items, especially those that are used for food and beverage containers. For this reason, structural analogs to BPA, including bisphenol F, have been utilized to reduce BPA content while ensuring product quality (Lehmler et al., 2018).

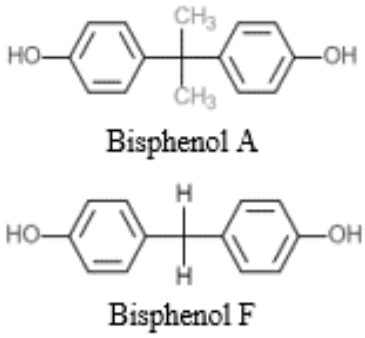

Figure 1: Structures of BPA and BPF

Bisphenol F (BPF) is a structural analog to BPA that replaces the two methyl groups at the central carbon with two hydrogen atoms, as seen in **Figure 1**. BPF has since replaced BPA in many epoxy-resin and coating materials in processes requiring high-strength bindings in addition to many products directly utilized by the consumer, including dental sealants, varnishes, and food packaging (Rochester & Bolden, 2015). Although this is meant to reduce the quantity of harmful chemicals introduced into the environment and in consumer products, several studies have investigated the EDC activity of BPF. These studies have suggested that BPF exhibits effects to the same order of magnitude of BPA and, in some instances, acts as a more powerful EDC than BPA in certain body systems (Eladak et al., 2015;

Rochester & Bolden, 2015). BPF is found in human urine at levels comparable to BPA, and this quantity is expected to rise due to the continuing increase of BPF in products (Rochester & Bolden, 2015; Lehmler et al., 2018). Thus, in order to protect human and environmental health, the systemic effects of BPF exposure must be investigated in the coming years.

Zebrafish (*Danio rerio*) are an effective model for vertebrate and human development. Zebrafish demonstrate a high gene conservation rate compared to humans and have been used in numerous toxicological studies (Yang et al., 2018). Zebrafish express proteins and molecular signaling pathways that are similar to humans (Yang et al., 2018). Experiments utilizing zebrafish have shed light on chemical interactions within vertebrates that can be extended to human health, including studies that have elucidated genes for Parkinson's (de Fonseca et al., 2013), examined effect of nicotine on proteins (Suen et al., 2013), and investigated the cardiovascular response to new pharmaceutical products (Lee et al., 2013).

The purpose of this study was to observe the effect of bisphenol F addition to water inhabited by zebrafish embryos on their cardiac output and stroke volume. I hypothesized that bisphenol F will decrease the overall cardiac output in zebrafish embryos and will lead to a higher death rate overall.

Materials and Methods:

Wild-type zebrafish (*Danio rerio*) housed at the University of Akron Research Vivarium were utilized as the breeding stock to obtain for this experiment. Standard housing conditions were maintained for adult zebrafish at a temperature of 28 ± 0.5°C. A breeding box was introduced the in the evening prior to the dark cycle; breeding occurs just prior to the beginning of the light cycle in adults. Embryos were collected, rinsed, and transferred to laboratory B235 for containment and exposures.

Embryos were exposed to three treatment groups in this experiment: dechlorinated water, dechlorinated water with the addition of ethanol, and dechlorinated water with the addition of ethanol and BPF. Following collection of embryos from breeding boxes, flasks containing the various treatment group solutions were prepared. Previous work found that 1% ethanol does not significantly increase mortality rates in zebrafish embryos (Duan et al., 2008). Control flasks were prepared using only dechlorinated water, ethanol-exposure flasks were prepared to a concentration of 1% ethanol, and BPF flasks were prepared to a concentration of 1% ethanol with a BPF concentration of 50 µg/L. Embryos (n = 58) were divided among treatment groups (n = 31 for BPF, n = 19 for control, and n = 18 for ethanol). After 24 hours post-exposure (hpe) and 48 hpe, five to ten second videos of each embryo heart were taken using an inverted microscope using at high speed camera at 125 frames per second. All embryos were euthanized prior to 72 hours post fertilization using 0.5% MS-222. ImagePro software was used to measure ventricle length and width at systole and diastole in order to find ventricle volume. Ventricle volume was determined according to the method of (Bagatto & Burggren, 2005) using the formula for a prolate spheroid

$$\frac{4\pi a b^2}{3}$$

where *a* was the length of the longitudinal axis of the ellipse and *b* was the length of the width of the ellipse. Stroke volume was found by calculating the difference between the systolic and diastolic volumes. Heart beat was measured by calculating the number of frames between five or ten beats of the heart and using camera frame rate to measure beats per minute. Cardiac output was determined by multiplying stroke volume by heart rate for each embryo. As a result of using several treatment flasks, analysis of variance (ANOVA) and Tukey HSD analysis were performed to determine statistical significance ($p < 0.05$) among treatment groups.

Results:

As seen in **Figure 2** and **Figure 3**, no statistical difference was found between any treatment groups at either 24 hpe ($p = 0.616$ and $p = 0.1813$) or 48 hpe ($p = 0.2409$ and $p = 0.0551$).

The BPF treatment group was found to have a statistically lower mean stroke volume at 24 hpe ($p < 0.001$) with a mean of 9.51±0.86 nL. Compared to stroke volume of control embryos at 15.71±1.14 nL and ethanol at 14.70±1.14 nL, the BPF group exhibited a 35% reduction. However, as seen in **Figure 4**, at 48 hpe, the ethanol group had a significantly lower mean stroke volume of 21.43±4.98 nL. This group experienced a 42% reduction in stroke volume compared to the BPF treatment group at 37.53±4.07 nL and the control treatment group at 37.09±3.32 nL ($p = 0.0274$).

As seen in **Figure 5**, at 24 hpe, the BPF treatment group did not statistically differ from either treatment group, but the ethanol group had a mean heart rate with a mean of 96.80±2.84 bpm, 12% higher than to the control group with a mean of 85.67±2.84 bpm ($p = 0.0177$). At 48 hpe, the ethanol treatment had a significantly lower mean heart rate with 78.25±6.14 bpm while BPF had a mean of 143.33±5.01 bpm and control mean a mean of 137.28±4.09 bpm ($p < 0.0001$).

The BPF treatment group had a statistically lower mean cardiac output at 24 hpe as compared to the other treatment groups ($p = 0.0014$). As seen in **Figure 6**, mean cardiac outputs were 43% lower in the BPF group at 907±94 nL/min while mean cardiac outputs for other groups were 1394±124 nL/min for control and 1414±124 nL/min for ethanol. At 48 hpe, the ethanol group was significantly lower than the other groups with a mean of 2127±752 nL/min as compared to 5414±614 nL/min and 5109±501 nL/min for BPF and control groups, respectively

(p = 0.0032). The mean cardiac output of the ethanol group was 86% lower than the other treatment groups.

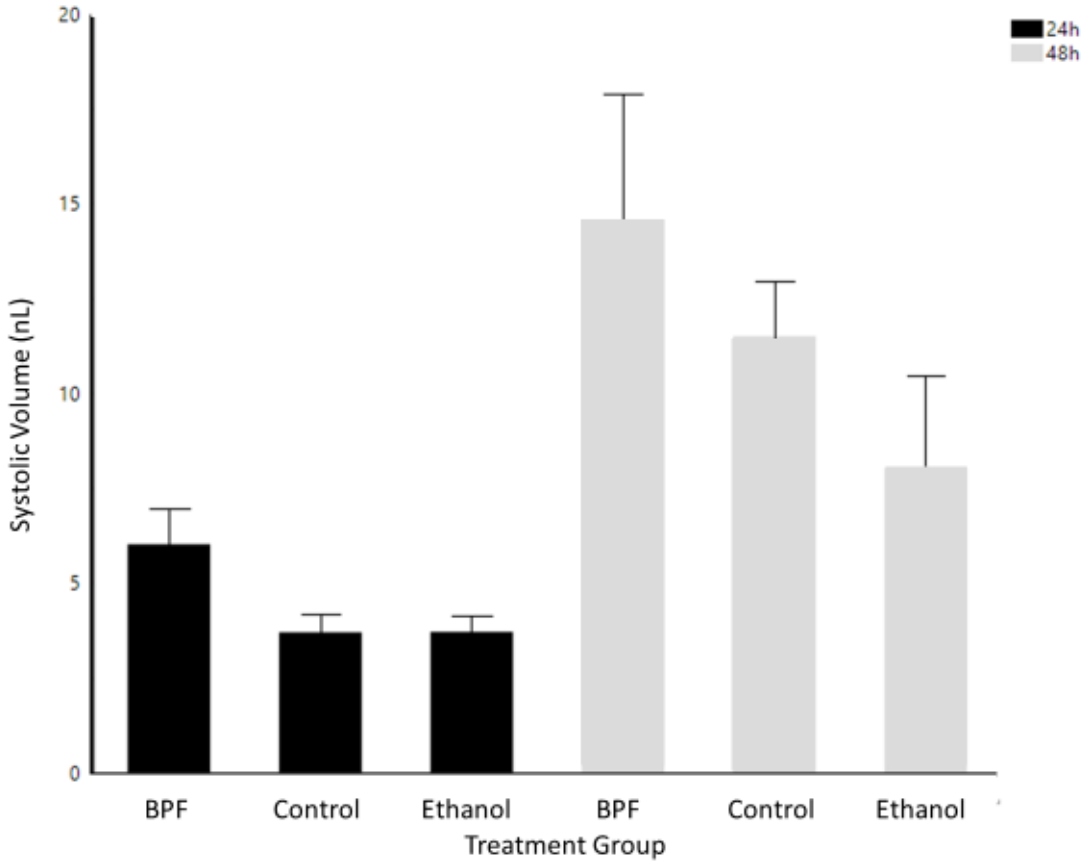

Figure 2 displays the mean systolic volume in nanoliters of each treatment group at 24 and 48 hpe. No significant difference was found among any of the treatment groups at either developmental stage (p = 0.616 and p = 0.2409, respectively). Error bars illustrate standard error. Samples sizes at 24 hpe were BPF (n = 31), control (n = 19), and ethanol (n = 18). At 48 hpe, they were: BPF (n = 20), control (n = 18), and ethanol (n = 10)

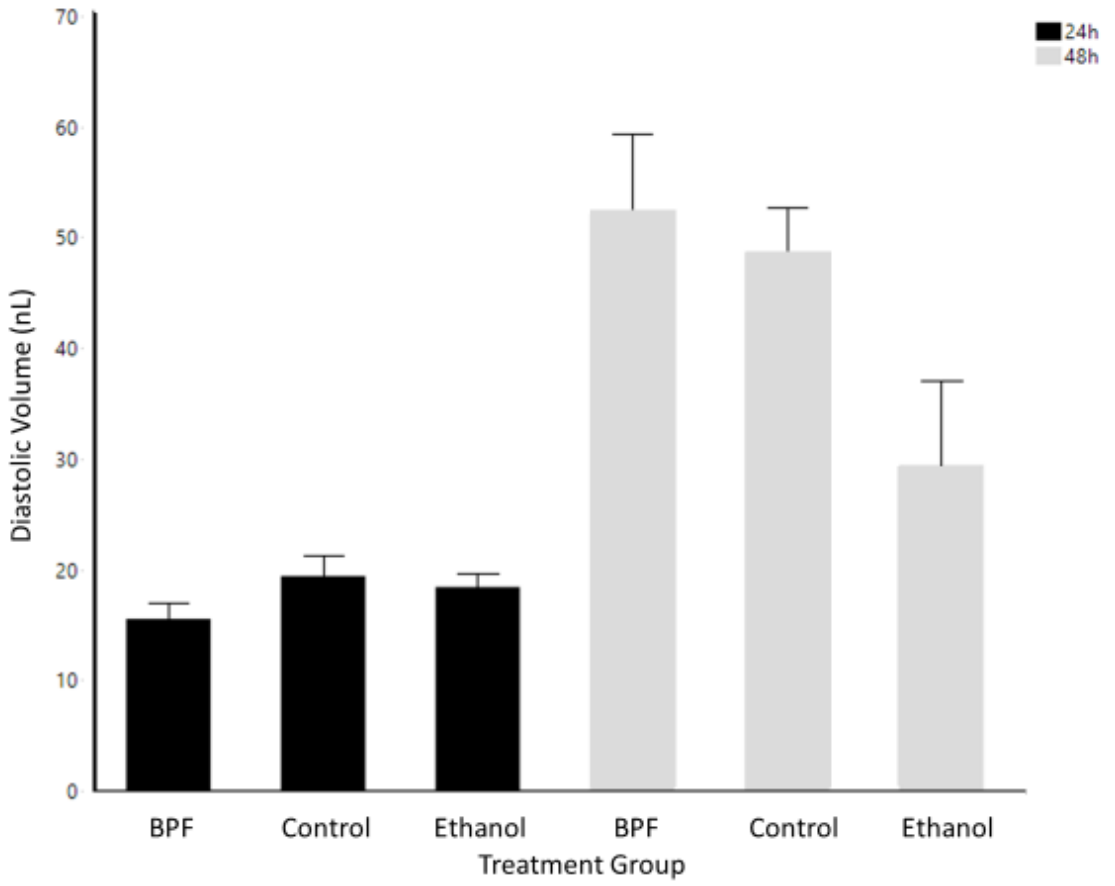

Figure 3 exhibits the mean diastole volume in nanoliters of each treatment group at 24 and 48 hpe. No significant difference was found among any of the treatment groups at either developmental stage ($p = 0.1813$ and $p = 0.0551$, respectively). Error bars illustrate standard error. Samples sizes at 24 hpe were BPF ($n = 31$), control ($n = 19$), and ethanol ($n = 18$). At 48 hpe, they were: BPF ($n = 20$), control ($n = 18$), and ethanol ($n = 10$)

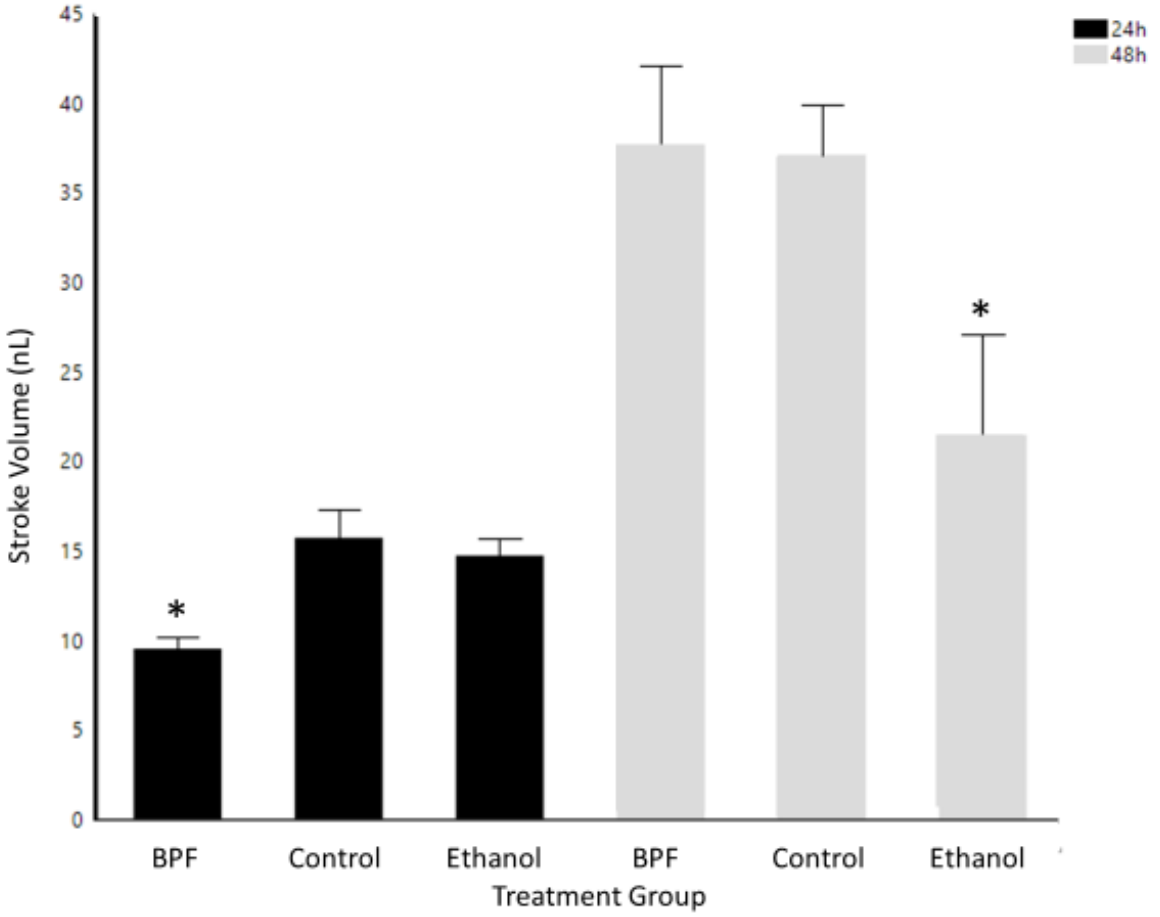

Figure 4 displays the mean stroke volume in nanoliters of each treatment group at 24 and 48 hpe. Significant differences were found among treatment groups at both developmental stages ($p < 0.001$ and $p = 0.0274$, respectively) as denoted by asterisks. Error bars illustrate standard error. Samples sizes at 24 hpe were BPF (n = 31), control (n = 19), and ethanol (n = 18). At 48 hpe, they were: BPF (n = 20), control (n = 18), and ethanol (n = 10)

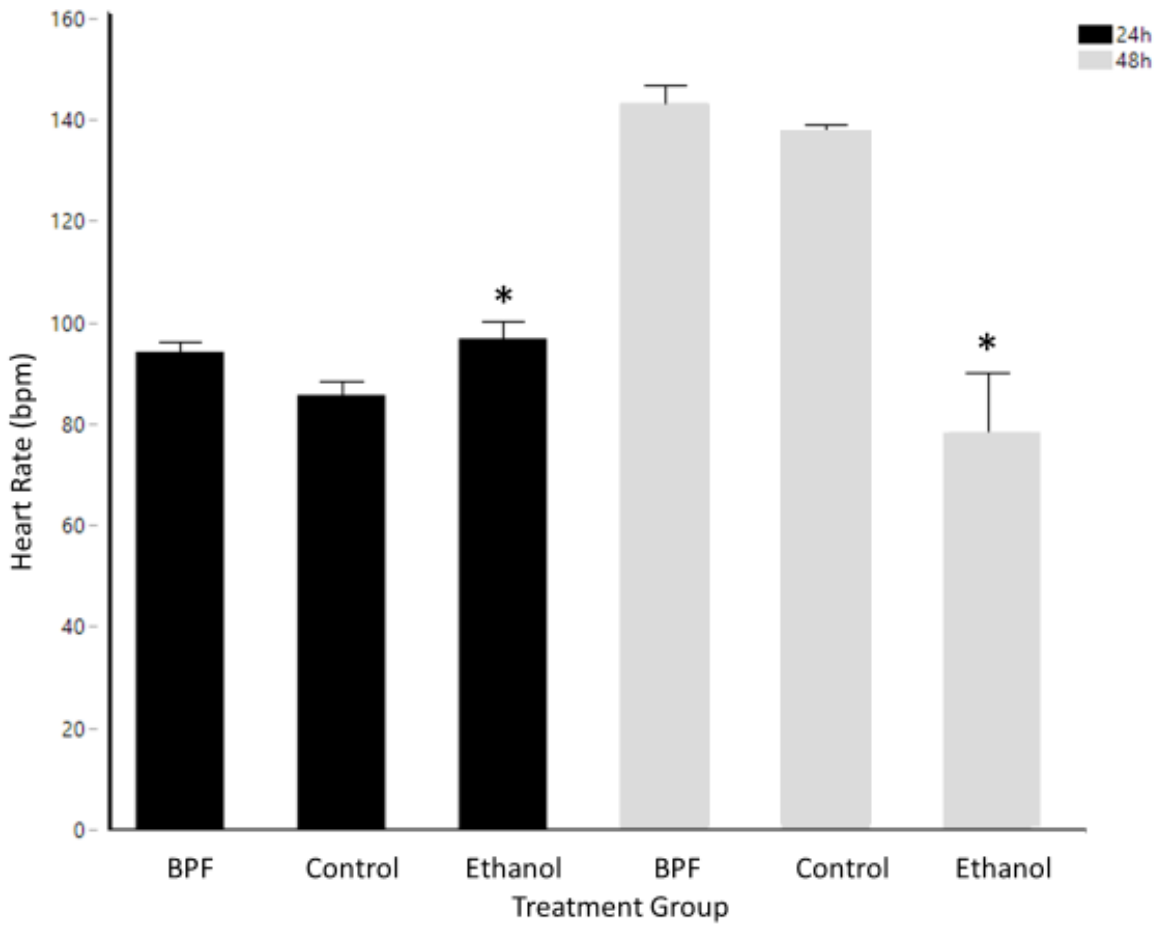

Figure 5 illustrates the mean heart rate in beats per minute of each treatment group at 24 and 48 hpe. Statistical differences were observed among treatment groups at 24 and 48 hpe ($p = 0.0177$ and $p < 0.0001$, respectively) as denoted by asterisks. Error bars illustrate standard error. Samples sizes at 24 hpe were BPF (n = 31), control (n = 19), and ethanol (n = 18). At 48 hpe, they were: BPF (n = 20), control (n = 18), and ethanol (n = 10)

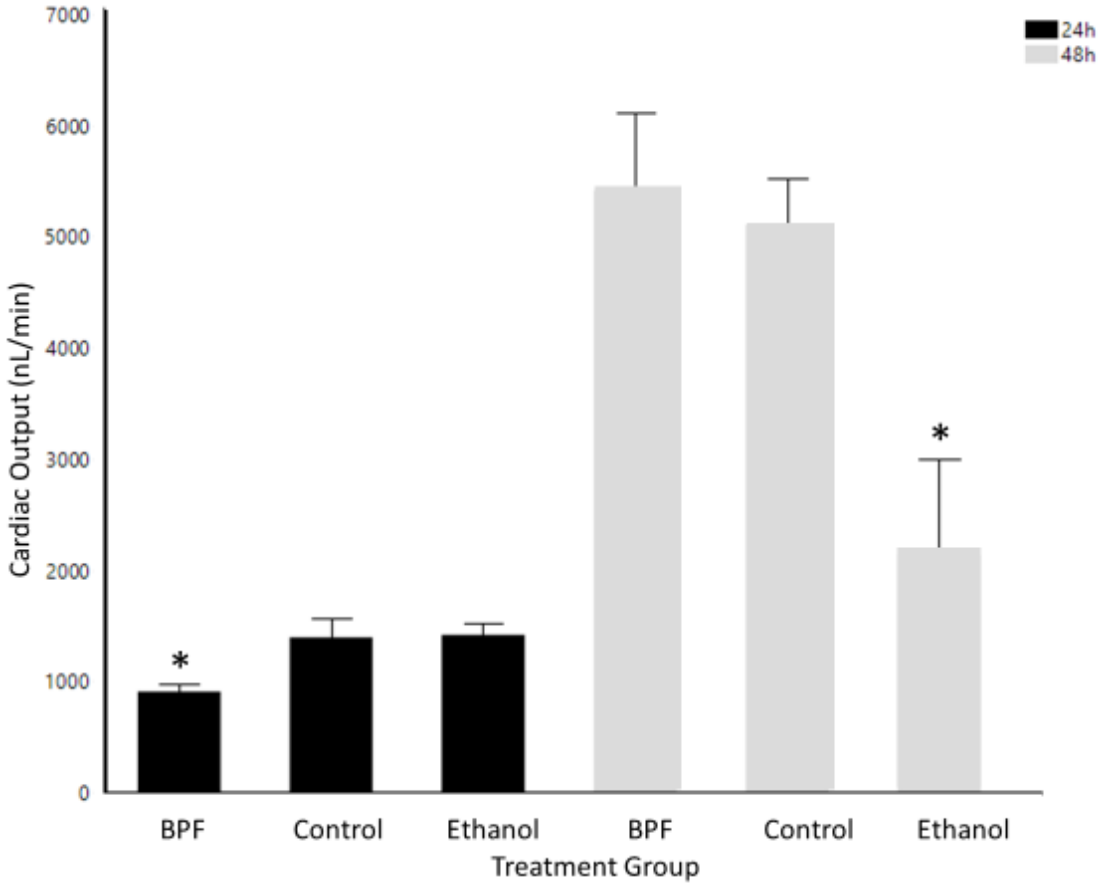

Figure 6 displays the mean cardiac outputs in nanoliters per minute of each treatment group at 24 and 48 hpe. Significant differences were found among treatment groups at both developmental stages ($p = 0.0014$ and $p = 0.0032$, respectively) as denoted by asterisks. Error bars illustrate standard error. Samples sizes at 24 hpe were BPF ($n = 31$), control ($n = 19$), and ethanol ($n = 18$). At 48 hpe, they were: BPF ($n = 20$), control ($n = 18$), and ethanol ($n = 10$)

Discussion:

At 24 hpe, the mean stroke volume of the BPF experimental group was statistically lower compared to both the control and ethanol groups ($p < 0.001$). **Figure 4** illustrates that the control and ethanol groups did not exhibit a significant difference while the BPF group had a lower mean stroke volume. Similarly, noted in **Figure 6**, as cardiac output is directly correlated to stroke volume, the BPF treatment group had a significantly lower mean cardiac outputs ($p =$

0.0014). This 35% reduction in stroke volume and cardiac output correlates to the results of other studies that found decreased cardiac parameters following exposure to BPA (Duan et al., 2008; Cypher et al., 2015). It also correlates with studies that have shown that the analog BPA causes a 300% increase in premature ventricular beats that would impede proper cardiac muscle function (Yan et al., 2011).

Mean heart rates for the ethanol treatment groups at 24 hpe were significantly higher than only the control group as seen in **Figure 5** ($p = 0.018$). The BPF treatment group did not differ significantly from either group. Some studies have found that heart rate does not differ under the addition of xenoestrogens (Soares et al., 2009) while others have demonstrated that BPA causes up to a 42% decrease in heart rate (Cypher et al., 2015). In this light, it is possible that ethanol may have influenced the cardiovascular rhythm of the embryos that caused an approximately 50% decrease in stroke volume and cardiac output parameters compared to controls.

Observations at 48 hpe produced a differing condition of results. The ethanol treatment group was observed to have a significantly lower value for all three parameters of mean stroke volume, heart rate, and cardiac output ($p = 0.0274$, 0.0001, and 0.0032, respectively). Conversely, the BPF and control groups exhibited almost no difference between their parameters as seen in **Figure 4, Figure 5,** and **Figure 6**. It must be noted that 70% mortality was observed in the ethanol treatment group at 48 hpe. In one subgroup, all members that were alive at 24 hpe were found dead, while in another subgroup, all embryos were found near death with bodily pigmentation beginning to darken substantially, an indicator of tissue and organism death. As such, heart rates of ethanol-treated zebrafish at 48 hpe may be skewed downward as cardiovascular function in the dying organisms was beginning to fail. As such, 1% ethanol did have a significant effect upon mortality and development of zebrafish embryos contrary to other

studies (Duan et al., 2008; Bilotta et al., 2004). Death rates of the BPF and control groups were between 6 and 10%.

Overall, these results may confirm the results of (Cypher et al., 2018) which found that cardiac parameters experience a significant decrease in the presence of hypoxia and a xenoestrogen; therefore, it is possible that experimental design did not provide adequate oxygen circulation. Ethanol is known to cause mortality in zebrafish embryos and hypoxia is a common environmental stressor for these organisms; thus, the combination of these factors may have increased mortality exponentially (Bilotta et al., 2004).

Future studies are needed to continue work on BPF to investigate its effect on cardiovascular parameters. Although studies have been conducted to demonstrate the effect of BPA on heart disease, few studies have been conducted on the specific effects of its analog BPF on the function of cardiac systems. Due to a low sample size (n = 58 among all treatment groups; n = 31 for BPF), observations on the statistical differences between treatment groups may exhibit some bias in this study. Larger sample sizes and repetitions of experiments should be conducted to verify the findings of this study and to shed more light on the problem of bisphenol A substitutes. As BPF continues to be used more ubiquitously in consumer products, more research must be done to ensure its safety.

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